

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference B0 41480 EE	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/NL 97/ 00474	International filing date (day/month/year) 21/08/1997	(Earliest) Priority Date (day/month/year)
Applicant DE STAAT DER NEDERLANDEN, VERTEGENWOORDIGD . et al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (see Box I).

2. ☐ Unity of invention is lacking (see Box II).

3. ☒ The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

☒ filed with the international application.

☐ furnished by the applicant separately from the international application.

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the title, ☒ the text is approved as submitted by the applicant

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

Figure No. ☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

Nederlandsch Octrooibureau

INGEK. 15 MRT 1999

Paraaf Bewerken

PCT

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:

DE BRUIJN, Leendert, C.
Nederlandsch Octrooibureau
Scheveningseweg 82, P.O. Box 29720
NL-2502 LS The Hague
PAYS-BAS

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 04 March 1999 (04.03.99)		IMPORTANT NOTICE
Applicant's or agent's file reference BO 41480 EE		
International application No. PCT/NL97/00474	International filing date (day/month/year) 21 August 1997 (21.08.97)	Priority date (day/month/year)
Applicant DE STAAT DER NEDERLANDEN, VERTEGENWOORDIGD DOOR DE MINISTER VAN WELZIJN, VOLKSGEZONDHEID EN CULTUUR et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU, BR, CN, EP, IL, JP, KP, KR, US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
AL, AM, AP, AT, AZ, BA, BB, BG, BY, CA, CH, CU, CZ, DE, DK, EA, EE, ES, FI, GB, GE, GH, HU, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, OA, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
04 March 1999 (04.03.99) under No. WO 99/10497

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer J. Zahra Telephone No. (41-22) 338.83.38
--	---

09/486073

y for the Elected Office (EO/US)

5060 PCT/NL97/00474

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

DE BRUIJN, Leendert, C.
Nederlandsch Octrooibureau
Scheveningseweg 82, P.O. Box 29720
NL-2502 LS The Hague
PAYS-BAS

Date of mailing (day/month/year) 22 February 2000 (22.02.00)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference BO 41480 EE	
International application No. PCT/NL97/00474	International filing date (day/month/year) 21 August 1997 (21.08.97)

1. The following indications appeared on record concerning: <input checked="" type="checkbox"/> the applicant <input checked="" type="checkbox"/> the inventor <input type="checkbox"/> the agent <input type="checkbox"/> the common representative	
Name and Address STEEGHS, Liana, Juliana, Josephine, Margret Arthur van Schendelstraat 539 NL-3511 NP Utrecht Netherlands	State of Nationality NL State of Residence NL Telephone No. Facsimile No. Teleprinter No.
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input type="checkbox"/> the person <input checked="" type="checkbox"/> the name <input checked="" type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence	
Name and Address STEEGHS, Liana, Juliana, Josephine, Margriet Arthur van Schendelstraat 539 NL-3511 MP Utrecht Netherlands	State of Nationality NL State of Residence NL Telephone No. Facsimile No. Teleprinter No.
3. Further observations, if necessary:	
4. A copy of this notification has been sent to: <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the designated Offices concerned <input type="checkbox"/> the International Searching Authority <input checked="" type="checkbox"/> the elected Offices concerned <input type="checkbox"/> the International Preliminary Examining Authority <input type="checkbox"/> other:	


The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer R. Raissi Telephone No.: (41-22) 338.83.38
---	---

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference BO 41480		FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/NL97/00474	International filing date (day/month/year) 21/08/1997	Priority date (day/month/year) 21/08/1997	
International Patent Classification (IPC) or national classification and IPC C12N15/31			
Applicant DE STAAT DER NEDERLANDEN, VERTEGENWOORDIGD . et al			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 4 sheets, including this cover sheet.</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of 5 sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application 			
Date of submission of the demand 18/03/1999		Date of completion of this report	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer Halle, F Telephone No. +49 89 2399 8537	



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/NL97/00474

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

3-16 as originally filed

1,2 as received on 15/10/1999 with letter of 15/10/1999

Claims, No.:

1-18 as received on 15/10/1999 with letter of 15/10/1999

Drawings, sheets:

1/5-5/5 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/NL97/00474

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-18
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-18
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-18
	No:	Claims	

2. Citations and explanations

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/NL97/00474

Section V

Having regard to the prior art, the subject-matter of claims 1-18 appears to be novel and to involve an inventive step.

The prior art document WO 97 25061 (D1) discloses LPS-deficient Gram-negative bacteria and their use in vaccines; these bacteria are LPS-deficient in levels of the myristic acid moiety. In the disclosure of the prior art document WO 97 19688 (D2), it is referred to mutated Gram-negative bacterial pathogens having reduced toxicity due to a modification in the region of the fatty acyl chains of lipid A of the LPS molecule. Thus the prior art disclosures refer to a deficiency in the LPS molecule in order to obtain a de-myristolated LPS which may be used as an LPS antagonist of LPS-mediated activation (D1) or to obtain a bacterial pathogen with reduced toxicity and retaining the antigenicity of the corresponding wild type endotoxin LPS. In fact, the prior art is suggesting the necessity of using vaccine formulations containing modified LPS molecules rather than using formulations free of LPS. Therefore, the subject-matter claimed is not anticipated by the prior art and is not obvious to a person skilled in the art.

Novel mutants of gram negative mucosal bacteria and application thereof in vaccines.

Summary of the invention

5 We found that contrary to previous findings with *E. coli* it is possible to inactivate the early stage of lipid A synthesis of mucosal gram negative bacteria without compromising cell viability. In particular the *lpxA* gene in *Neisseria meningitidis* was mutated without compromising cell viability. The resulting *lpxA* knockout mutants were found to be completely LPS-deficient. The major outer membrane proteins (OMPs) were
10 detected in normal amounts. Also, an outer membrane could be discerned in electron micrographs of ultrathin sections. To our knowledge, this was the first instance of a viable Gram-negative bacterial mutant completely lacking in LPS.

The finding provides important implications for our understanding of structure and biogenesis of the outer membrane. On a practical level, the availability of LPS-deficient
15 mutants of pathogenic mucosal bacteria such as *N. meningitidis* opens up new avenues to vaccine development. It enables easy isolation of endotoxin-free purified proteins, outer membranes or even whole-cell preparations for use in immunisation.

Background information

20 Lipopolysaccharide (LPS) constitutes the outer monolayer of the outer membrane of Gram-negative bacteria. As such it forms an important component of the outer membrane and has been considered relevant for vaccine purposes (Verheul et al, 1993). The membrane-anchoring lipid A part is responsible for the well-known endotoxin activity of the molecule (Zähringer et al., 1994).

25 Such endotoxin activity is undesirable in vaccines. Currently some preparations to be used in vaccines are subjected to rigorous, time consuming and costly purification procedures in order to remove this endotoxin activity prior to their being suitable for use as a vaccine. This allows higher doses due to reduced toxicity. However, drastic purification methods can easily lead to denaturation of protein antigens which need to retain their native
30 conformation in order to induce an appropriate immune response. To date Group A and C polysaccharide vaccines are available which have been rendered substantially free of lipopolysaccharide by means of purification. To date however no whole cell vaccines substantially free of LPS nor OMP vaccines substantially free of LPS have been produced.

AMENDED SHEET

The following references provide details of processes used to date in order to avoid LPS in pharmaceutical products Akers (1985), Gabler (1987) and the European Pharmacopoeia, 2nd edition "test for non-pyrogenicity". Specifically WHO Tech. Rep. Ser 594:50 1976 deals with the requirements for a meningococcal polysaccharide vaccine.

5 Mutants with defects in LPS biosynthesis have been described for many bacterial species however none of these have been considered as candidates for a vaccine free of the endotoxic LipidA. All viable mutants retain a minimal lipid A - KDO structure which is the first part to be synthesised (Raetz, 1990) in LPS synthesis. Thus they would not be suitable to overcome the above-mentioned problem facing vaccine producers. Above all, only conditionally lethal mutations have been reported for genes involved in early steps of lipid A biosynthesis in *Escherichia coli* (Raetz 1990). These mutants have a mutation in genes involved in early steps of lipid A biosynthesis. This finding suggested that this part of the LPS molecule is in fact essential for bacterial growth. As such this finding would be considered dissuasive by persons skilled in the art of producing vaccines of mutating genes associated herewith as the resulting mutant would not grow.

10 Inhibitors of lipid A biosynthesis have also been found to lead to rapid loss of cell viability in *E. coli* and several other bacteria (Onishi et al, 1996) thus supporting the above-mentioned hypothesis concerning the essential nature of lipidA biosynthesis.

20 In addition models for biogenesis of OMPs have been proposed in which their correct folding and targeting is dependent on LPS (Sen and Nikaido, 1991; Reid et al., 1990; Laird et al., 1994; de Cock and Tommassen, 1996).

WO 97/25061 discloses mutants of gram-negative bacteria having a form of LPS deficient in levels of myristic acid moiety, in which the *lpxF* gene is inhibited.

25 WO 97/19688 describes mutants of gram-negative bacteria producing less toxic LPS as a result of a mutation in the *htrB* gene.

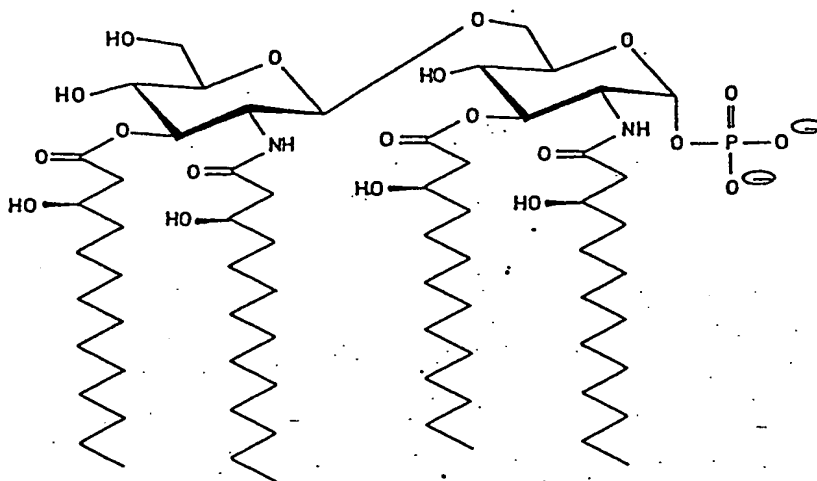
30 We previously cloned the *lpxA* gene from *Neisseria meningitidis* which encodes the enzyme UDP-GlcNAc acyltransferase required for the first step in lipid A biosynthesis (Steeghs et al. 1997). While attempting to alter the fatty acyl specificity of this enzyme by constructing an *E. coli-N. meningitidis* hybrid *lpxA* gene, we made the unexpected discovery forming the basis of the subject invention.

Detailed description of the invention.

AMENDED SHEET

CLAIMS

1. Gram-negative mutant of a mucosal bacterium, comprising a mutation such that it is viable, is capable of OMP formation and lacks endotoxic lipopolysaccharide (LPS), the mutant being free of LPS.
2. Gram-negative mutant according to claim 1 comprising a mutation such that it is free of Lipid A.
3. Gram-negative mutant according to claim 1 or 2, said bacterium being selected from the group comprising diplococci.
4. Gram-negative mutant according to any one of claims 1-3, said bacterium being selected from the group comprising gonococci, e.g. *N. gonorrhoeae*.
5. Gram-negative mutant according to any one of claims 1-3, said bacterium being selected from the group comprising meningococci e.g. *N. meningitidis*.
6. Gram-negative mutant according to claim 1 or 2, said bacterium being selected from the group comprising *Bordetella*, e.g. *Bordetella pertussis*.
7. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least one gene associated with Lipid A biosynthesis.
8. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least one gene associated with the early stage of Lipid A biosynthesis, said early stage being prior to formation of the following structure



AMENDED SHEET

9. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least one gene selected from the group comprising *lpxA*, *lpxD* and *lpxB*.

10. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least the gene *lpxA*.

11. Attenuated live vaccine against a gram-negative mucosal bacterium, said vaccine comprising a mutant according to any one of claims 1-10 as an active component and a pharmaceutically acceptable carrier.

12. Whole cell vaccine against a gram-negative mucosal bacterium, said vaccine comprising a mutant according to any one of claims 1-10 as an active component and a pharmaceutically acceptable carrier.

13. OMP vaccine against a gram-negative mucosal bacterium said vaccine comprising OMP derived from a mutant according to any one of claims 1-10 as an active component and a pharmaceutically acceptable carrier.

14. Vaccine according to any one of claims 11-13 further comprising an adjuvant.

15. Vaccine according to any one of claims 11-14 said vaccine being substantially free of endotoxic LPS, wherein substantially free is defined as LPS-free according to the Limulus assay.

16. A method of producing LPS-free vaccine comprising application of a mutant according to any one of claims 1-10 and/or a part derived from said mutant as active component of a vaccine in a manner known per se for preparing vaccine formulations, said method being free of measures to remove LPS by purification.

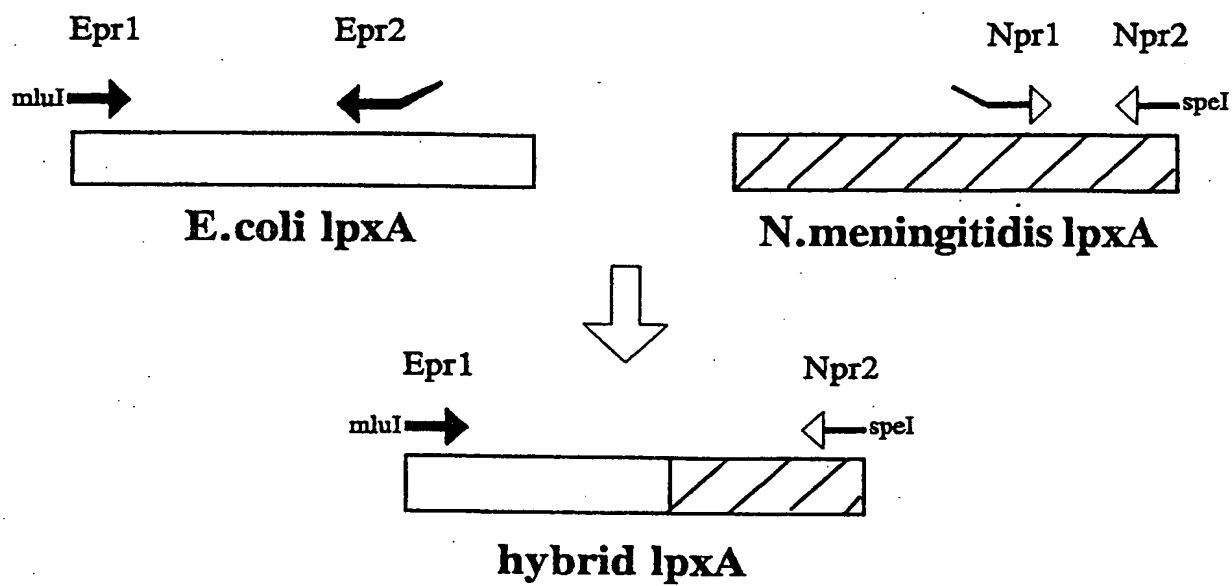
17. A method of producing LPS-free OMP comprising culturing a mutant according to any one of claims 1-10 and deriving an OMP comprising fraction from said culture in a manner known per se for isolating protein from bacterial culture, said method being free of

additional measures to remove LPS from said culture or OMP comprising fraction.

18. OMP which is free of LPS.

AMENDED SHEET

fig -1



2/5

fig-2

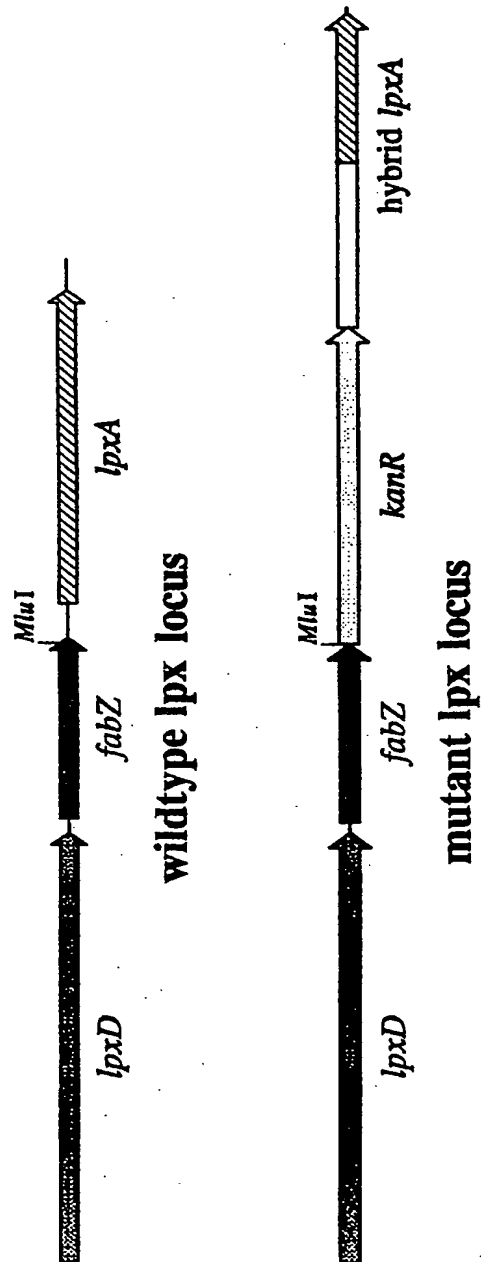
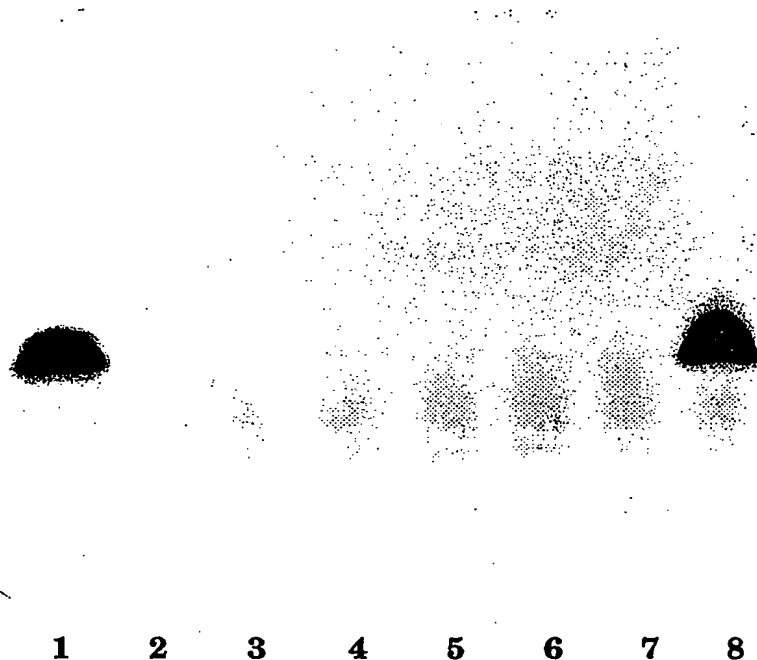


Figure 3

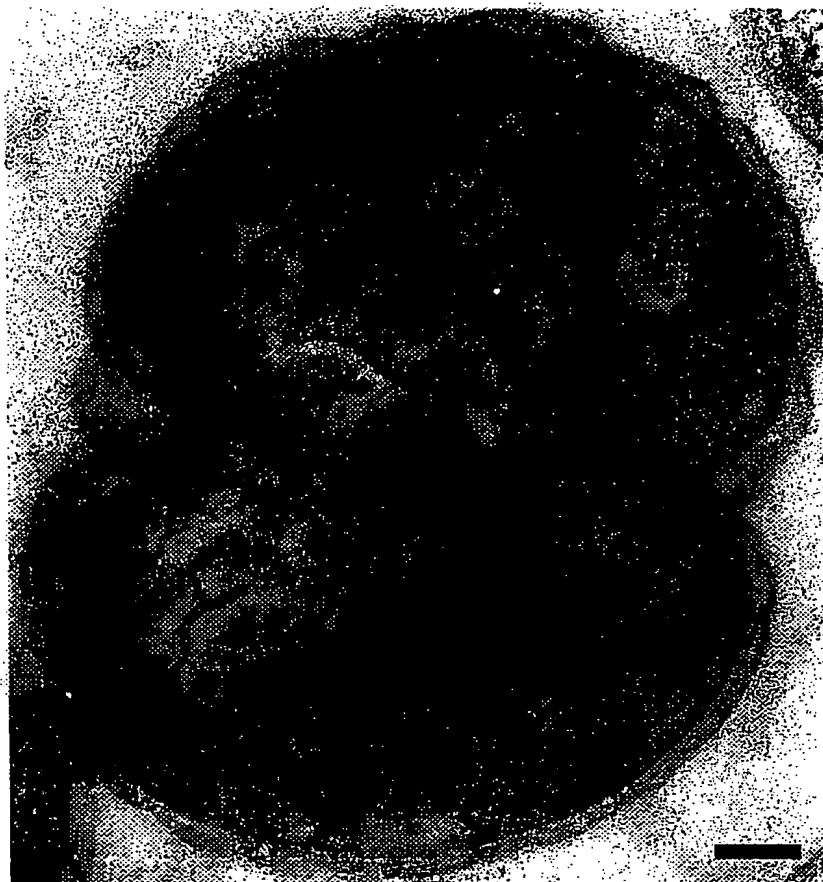


4/5

Figure 4



Figure 5



INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 97/00474

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N1/20 A61K39/02 A61K39/095 C07K14/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25061 A (SQUIBB BRISTOL MYERS CO ; SOMERVILLE JOHN E JR (US); DARVEAU RICHAR) 17 July 1997 see page 3, line 32 - page 5, line 20 ---	1,7,8, 12,13, 15,16
X	WO 97 19688 A (UNIV IOWA RES FOUND ;UNIV CALIFORNIA (US); AMERICAN CYANAMID CO (U) 5 June 1997 see page 4, line 10 - page 5, line 5 see page 6, line 29 - page 7, line 7 ---	1,4-6,8, 12,13, 15,16
X	EP 0 624 376 A (AMERICAN CYANAMID CO) 17 November 1994 see page 2, line 45 - page 3, line 21 ---	14-17,20
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

6 May 1998

Date of mailing of the international search report

26/05/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

Inter: 1al Application No
PCT/NL 97/00474

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>STEEGHS ET AL: "ISOLATION AND CHARACTERIZATION OF THE NEISSERIA MENINGITIDIS LPXD-FABZ-LPXA GENE CLUSTER INVOLVED IN LIPID A BIOSYNTHESIS" GENE, vol. 190, no. 2, 6 May 1997, pages 263-270, XP002064116 ✓ cited in the application see the whole document</p> <p>---</p>	
A	<p>ODEGAARD ET AL: "SHORTENED HYDROXYACYL CHAINS ON LIPID A OF ESCHERICHIA COLI CELLS EXPRESSING A FOREIGN UDP-N-ACETYLGLUCOSAMINE O-ACYLTRANSFERASE" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 32, 8 August 1997, pages 19688-19696, XP002064117 ✓ see page 19688 see abstract</p> <p>---</p>	
A	<p>RAETZ ET AL: "BIOCHEMISTRY OF ENDOTOXINS" ANNUAL REVIEWS OF BIOCHEMISTRY, vol. 59, 1990, pages 129-170, XP002064118 ✓ cited in the application see page 147, paragraph 4 - page 148, paragraph 3</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter: 1st Application No

PCT/NL 97/00474

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725061 A	17-07-97	NONE	
WO 9719688 A	05-06-97	AU 1124697 A	19-06-97
EP 0624376 A	17-11-94	CA 2123355 A	14-11-94
		JP 8019396 A	23-01-96



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/31, 1/20, A61K 39/02, 39/095, C07K 14/22	A1	(11) International Publication Number: WO 99/10497 (43) International Publication Date: 4 March 1999 (04.03.99)
(21) International Application Number: PCT/NL97/00474 (22) International Filing Date: 21 August 1997 (21.08.97) (71) Applicant (for all designated States except US): DE STAAT DER NEDERLANDEN, VERTEGENWOORDIGD DOOR DE MINISTER VAN WELZIJN, VOLKSGEZONDHEID EN CULTUURP.O. Box 5406 [NL/NL]; NL-2280 HK Rijswijk (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): VAN DER LEY, Peter, André [NL/NL]; Veeartsenijstraat 211, NL-3572 DJ Utrecht (NL). STEEGHS, Liana, Juliana, Josephine, Margret [NL/NL]; Arthur van Schendelstraat 539, NL-3511 NP Utrecht (NL). (74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: NOVEL MUTANTS OF GRAM NEGATIVE MUCOSAL BACTERIA AND APPLICATION THEREOF IN VACCINES		
(57) Abstract <p>It is possible to inactivate the early stage of lipid A synthesis of mucosal gram negative bacteria without compromising cell viability. In particular the <i>lpxA</i> gene in <i>N. meningitidis</i> was mutated and resulting <i>lpxA</i> knockout mutants were found to be completely lipopolysaccharide(LPS)-deficient. The major outer membrane proteins (OMPs) were detected in normal amounts. The finding provides important implications for understanding of structure and biogenesis of the outer membrane. On a practical level, the availability of LPS-deficient mutants of pathogenic mucosal bacteria such as <i>N. meningitidis</i> opens up new avenues to vaccine development. It enables easy isolation of endotoxin-free purified proteins, outer membranes or even whole-cell preparations for use in immunisation.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Novel mutants of gram negative mucosal bacteria and application thereof in vaccines.

Summary of the invention

5 We found that contrary to previous findings with *E.coli* it is possible to inactivate the early stage of lipid A synthesis of mucosal gram negative bacteria without compromising cell viability. In particular the *lpxA* gene in *N.meningitidis* was mutated without compromising cell
10 viability. The resulting *lpxA* knockout mutants were found to be completely LPS-deficient. The major outer membrane proteins (OMPs) were detected in normal amounts. Also, an outer membrane could be discerned in electron micrographs of ultrathin sections. To our knowledge, this was the first instance of a viable Gram-negative bacterial mutant completely
15 lacking in LPS.

The finding provides important implications for our understanding of structure and biogenesis of the outer membrane. On a practical level, the availability of LPS-deficient mutants of pathogenic mucosal bacteria such as *N.meningitidis* opens up new avenues to vaccine
20 development. It enables easy isolation of endotoxin-free purified proteins, outer membranes or even whole-cell preparations for use in immunisation.

Background information

25 Lipopolysaccharide (LPS) constitutes the outer monolayer of the outer membrane of Gram-negative bacteria. As such it forms an important component of the outer membrane and has been considered relevant for vaccine purposes (Verheul et al 1993). The membrane-anchoring lipid A
30 part is responsible for the well-known endotoxin activity of the molecule (Zähringer et al., 1994).

Such endotoxin activity is undesirable in vaccines. Currently some preparations to be used in vaccines are subjected to rigorous, time consuming and costly purification procedures in order to remove this
35 endotoxin activity prior to their being suitable for use as a vaccine. This allows higher doses due to reduced toxicity. However, drastic purification methods can easily lead to denaturation of protein antigens which need to retain their native conformation in order to induce an appropriate immune response. To date Group A and C polysaccharide

vaccines are available which have been rendered substantially free of lipopolysaccharide by means of purification. To date however no whole cell vaccines substantially free of LPS nor OMP vaccines substantially free of LPS have been produced. The following references provide details of processes used to date in order to avoid LPS in pharmaceutical products Akers (1985), Gabler (1987) and the European Pharmacopoeia, 2nd edition "test for non-pyrogenicity". Specifically WHO Tech. Rep. Ser 594:50 1976 deals with the requirements for a meningococcal polysaccharide vaccine.

Mutants with defects in LPS biosynthesis have been described for many bacterial species however none of these have been considered as candidates for a vaccine free of the endotoxic LipidA. All viable mutants retain a minimal lipid A - KDO structure which is the first part to be synthesized (Raetz, 1990) in LPS synthesis. Thus they would not be suitable to overcome the abovementioned problem facing vaccine producers. Above all, only conditionally lethal mutations have been reported for genes involved in early steps of lipid A biosynthesis in *Escherichia coli* (Raetz 1990). These mutants have a mutation in genes involved in early steps of lipid A biosynthesis. This finding suggested that this part of the LPS molecule is in fact essential for bacterial growth. As such this finding would be considered dissuasive by persons skilled in the art of producing vaccines of mutating genes associated herewith as the resulting mutant would not grow.

Inhibitors of lipid A biosynthesis have also been found to lead to rapid loss of cell viability in *E.coli* and several other bacteria (Onishi et al, 1996) thus supporting the abovementioned hypothesis concerning the essential nature of lipidA biosynthesis.

In addition models for biogenesis of OMPs have been proposed in which their correct folding and targeting is dependent on LPS (Sen and Nikaido, 1991; Reid et al., 1990; Laird et al., 1994; de Cock and Tommassen, 1996).

We previously cloned the *lpxA* gene from *Neisseria meningitidis* which encodes the enzyme UDP-GlcNAc acyltransferase required for the first step in lipid A biosynthesis (Steeghs et al. 1997). While attempting to alter the fatty acyl specificity of this enzyme by constructing an *E.coli-N.meningitidis* hybrid *lpxA* gene, we made the unexpected discovery forming the basis of the subject invention.

Detailed description of the invention.

The isolation of the *N. meningitidis* *lpxA* gene involved in lipid A biosynthesis has recently been reported (Steeghs et al., 1997). The deduced amino acid sequence of the LpxA protein showed homology to the *E.coli* acyltransferase responsible for adding the O-linked 3-OH myristoyl chain to UDP-N-acetylglucosamine, which is the first committed step in the lipid A biosynthetic pathway (Anderson and Raetz, 1987; Coleman and Raetz, 1988). Based on this homology and a comparison of the *E.coli* and *N.meningitidis* lipid A structures it is expected that the meningococcal *lpxA* gene encodes an acyltransferase with 3-OH lauroyl specificity (Kulshin et al., 1992). The basis of the different fatty acid specificity might conceivably be located in the characteristic hexapeptide repeat motif of these acyltransferases which has been determined to play a crucial role in the folding of the *E.coli* protein (Vuorio et al., 1994; Raetz and Roderick, 1995). In an attempt to verify this hypothesis we constructed a hybrid *lpxA* gene in which the meningococcal N-terminal β -helix domain containing the hexapeptide repeat motif was replaced by the corresponding part of *E.coli* *lpxA*, followed by transformation and allelic replacement of this construct to *N. meningitidis* H44/76. The experimental data for this are provided in the examples (in particular example 1).

The results demonstrated that strain H44/76[pHBK30] is a viable LPS-deficient mutant. The most likely explanation for this surprising discovery seemed to be that the hybrid *lpxA* gene had become inactive, either because of disrupted transcription/translation in our construct, or else because the hybrid protein as expressed had lost its enzymatic activity. To discern this, we constructed an *lpxA* knockout mutant. The results demonstrated once more that blocking of the lipid A biosynthesis pathway in *N. meningitidis* strain H44/76 leads to viable LPS-deficient mutants.

This is the first report of a viable Gram-negative bacterial mutant completely deficient in LPS. It has the following implications:

(1) Surprisingly (in view of the above mentioned view of the essential nature of lipidA biosynthesis for cell viability), it is possible for some gram negative bacteria to make an outer membrane without any LPS yet remain viable. Although our results do not exclude an involvement of LPS in the OMP forming process, they do demonstrate that it obviously cannot be essential. It should be very interesting to study the structure of the outer membrane in the *lpxA* mutant in more detail.

(2) In *E.coli*, all mutations affecting the early steps of lipid

A biosynthesis that have been described are lethal when expressed. The fact that this is not the case in Meningococci opened up the question which organism is typical in this respect, and what causes this difference. Conceivably, it is related to a different LPS-OMP interaction, which is also suggested by the observation that whereas deep-rough LPS mutants of *E.coli* and *Salmonella typhimurium* show a reduced expression of the major OMPs (Koplow and Goldfine, 1974; Ames et al., 1974), a comparable heptose-deficient *rfaC* mutant of *N.meningitidis* was found to have normal expression of the class 1 and 3 porins (Hamstra and van der Ley, unpublished).

We postulate that mucosal gram negative bacteria can in an analogous manner be mutated thereby becoming free of endotoxic LPS. Subsequently enabling development of LPS free whole cell or acellular vaccines such as OMP vaccines. The basis for this postulation is found in the knowledge available to the skilled person concerning the Lipid A biosynthesis in mucosal gram negative bacteria. Figure 6 e.g. as derived from Raetz 1990 provides a diagram of the early steps in lipid A biosynthesis. It reveals the requirement of *lpxA* and *lpxB* as enzymes required in the early biosynthesis. The enzyme *lpxD* is also known to be involved (Steeghs et al 1997). Knowledge of the nucleic acid sequences encoding these genes is available to the skilled person (Steeghs et al 1997). Subsequently mutating one or more of the genes encoding the enzymes involved in the early stages of LipidA biosynthesis is possible. Figure 6 shows the early stages; preferably the mutation will arise such that no stage leading past the *lpxB* stage is reached as these products already closely resemble Lipid A structure. Preferably the mutation will arise as early as possible in the biosynthesis pathway. In most cases the genes encoding *lpxA*, *lpxB* and *lpxD* are clustered. Steeghs et al provides references disclosing such details for *Escherichia coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Haemophilus influenzae* and *Rickettsia rickettsii*. Knowledge of the sequences of these microorganisms is thus available to the person skilled in the art and homologous sequences in other organisms can be found. Both *lpxA* and *lpxD* contain a characteristic hexapeptide repeat structure $[(I,V,L)GXXXX]_n$. The *lpxB* gene is generally cotranscribed with *lpxA* and as such can also be readily found. The cluster also comprises the *fabZ* gene which can also be used to ascertain the location of the gene cluster involved in Lipid A biosynthesis (Steeghs et al 1997). Steeghs et al provide the genbank number under which the *N.meningitidis* sequence data concerning the Lipid

A biosynthesis gene cluster is available i.e. U79481. The other references mentioned therein covering sequences of other organisms are also incorporated by reference.

There are two possibilities to mutate one or more genes associated with LipidA biosynthesis. Such mutation can be either such that enzyme is produced in an inactive form or such that the gene is mutated such that it is not expressed i.e. thereby forming a so called knockout mutant. The manners in which this objective can be achieved are numerous and will be readily available to a person skilled in the art of genetic engineering. Clearly by way of example of such a manner the analogous procedure to that employed in the examples can be used for different microorganisms and/or different enzymes known to be involved in the early stages of Lipid A biosynthesis. The principal of preparing knockout mutants in general is well known and can be applied to any of the genes encoding enzymes active in the early stages of Lipid A biosynthesis.

The subject invention comprises the mutants described above. In addition it comprises new vaccines made from such organisms or component parts thereof. Such new vaccines are free of Lipid A. Such vaccines can in fact be completely free of either active or inactive Lipid A. As a consequence the vaccines are free of LPS. The Limulus test can be applied as described herein to ascertain that a preparation is in fact free of LPS.

Thus a vaccine against a gram negative mucosal bacterium said vaccine being substantially free of LPS, wherein substantially free can be ascertained by the Limulus test, said vaccine comprising a microorganism according to the invention as disclosed above as active component falls within the scope of the invention. This is a so called whole cell vaccine. In addition a vaccine against a gram negative mucosal bacterium comprising one or more components of the aforementioned microorganism which is also substantially free of LPS as defined above is covered by the invention. In particular, an OMP comprising vaccine substantially free of LPS as defined above is covered by the invention.

A method of producing a vaccine against gram negative mucosal bacteria employing a microorganism according to the invention and/or parts derived therefrom as active component in a manner known per se for producing whole cell or acellular vaccines is covered by the invention as are the products of said method. The vaccines according to the invention will preferably be further characterised by the presence of an adjuvant

to enhance the immunogenic activity thereof. A number of adjuvants commonly used in vaccines are known. Any of these can be suitably applied. Any dosage form and additional components commonly used for vaccines, in particular meningococcal vaccines is suitable for the subject invention.

Particularly suited target microorganisms are diplococci and *Bordetella pertussis*. The diplococci comprise meningococci and gonococci. Examples of each category are *N.meningitidis* and *N. gonorrhoeae*. Numerous other organisms falling within this category are known from Bergeys Handbook of Determinative Bacteriology. These diplococci are structurally closely related and show the same gene structure. Both are interesting microorganisms from a vaccination view point as are a number of other microorganisms such as *Haemophilus influenzae* and *Moraxella catarrhalis*.

Clearly, the construction of *lpxA* knockout mutants can be attempted in other bacterial species known to have LipidA in their lipopolysaccharide.

(3) The availability of LPS-deficient mutants will allow new approaches to vaccine development against *N.meningitidis* and the closely related pathogen *N.gonorrhoeae*, as well as any other bacteria as mentioned above for which such mutants can be made and isolated. First, it will become much easier to purify OMPs or other cell surface components without contaminating endotoxin. Secondly the role of LPS in meningococcal outer membrane vesicle vaccines, e.g. as adjuvant or in stabilising OMP conformation (Verheul et al., 1993; Nakano and Matsuura, 1995; Poolman, 1995), can now be unequivocally determined and possibly taken over by a less toxic compound. Thirdly the use of inactivated whole cell vaccines can be investigated using endotoxin-free mutants according to the invention such as the *lpxA* mutants. Finally, the possibility to use LPS-deficient strains as live attenuated vaccines now arises.

The exact nature of the invention will be further elucidated with the following examples.

Example 1: Construction of an inactive *lpxA* gene in *N.meningitidis*

In two separate PCR reactions the *E.coli* and *N.meningitidis* part of the hybrid gene were amplified with the Epr1/Epr2 and Npr1/Npr2 primer, respectively (fig.1). The inside primers Epr2 and Npr1 were designed so that the ends of the products contain complementary

sequences. These products were mixed, denatured and reannealed in a second PCR in which the fused construct was amplified by the outside primers Epr1 and Npr2, having an *MluI* and *SpeI* site respectively (fig.1). The resulting PCR product was cloned and its sequence verified.

5 To test the activity of the hybrid *lpxA*, this gene was used to replace the original *lpxA* in the meningococcal chromosome (fig.2). For this purpose the 1.0 kb *MluI*/*SpeI* fragment carrying the wildtype *lpxA* gene in plasmid pLA19 (a pUC18 derivative with a 1.9 kb *lpxD-fabZ-lpxA* insert) was replaced by the similarly digested hybrid *lpxA* gene. Subsequently, a kanamycin-resistance cassette was ligated into the *MluI* site
10 located directly upstream of *lpxA*, resulting in the plasmid pHBK30.

Transformation of *N.meningitidis* H44/76 with linearized pHBK30 yielded kanamycin-resistant colonies after 24 hours of incubation. These mutants died when transferred to fresh GC plates with kanamycin (100
15 µg/ml).

By reducing the kanamycin concentration and screening of the resulting colonies by PCR amplification of *lpxA* hybrid-specific fragments we finally succeeded in the isolation of viable *kanR⁺* H44/76[pHBK30] transformants in which the chromosomal *lpxA* gene had been replaced by the
20 hybrid construct as shown in fig.2.

LPS of the H44/76[pHBK30] mutant and the wildtype strain was compared by Tricine-SDS-PAGE followed by a silver stain for carbohydrates (fig.3). Surprisingly, no LPS could be detected in the hybrid derivative by this method, even when higher amounts of cell lysates were loaded on
25 the gel.

To get more insight into the structure of the outer membrane of H44/76[pHBK30] a panel of LPS and OMP specific mAbs was tested in a whole cell ELISA (Table 1). The mutant strain did not bind any of the LPS-specific mAbs, whereas the OMP-specific mAbs showed similar binding
30 patterns for mutant and wildtype. This apparent OMP similarity was confirmed when OMCs of H44/76[pHBK30] and H44/76 were isolated and analysed by SDS-PAGE (fig.3). Both strains show equal amounts of the class 1, 3 and 4 OMP; in contrast to the wildtype, the mutant apparently also expresses a class 5 OMP.

35 Since LPS of H44/76[pHBK30] could not be detected with any of the methods described above, it became questionable whether it was present at all. Therefore, the mutant and wildtype strain were tested in a chromogenic Limulus (LAL) assay, with meningococcal medium as a negative control. This assay depends on activation of the clotting enzyme

cascade in amoebocyte lysate prepared from the horseshoe crab and is capable of detecting picogram quantities of endotoxin. The results of the LAL assay on cell suspensions showed no significant endotoxin activity for H44/76[pHBK30] over meningococcal medium (0.3 and 1.7 EU/ml, respectively), in contrast to 21.7×10^4 EU/ml for the wildtype.

Taken together, these results demonstrate that the initial attempt to replace the wildtype *lpxA* gene with the hybrid construct resulted in the isolation of what was apparently an LPS-deficient mutant. This was further confirmed by gas-chromatography/mass-spectrometry (GC-MS) analysis of fatty acids present in OMC preparations, which showed that the lipid A-specific 3-OH C12 was present only in trace amounts in the mutant. As this fatty acid is added in the first step of lipid A biosynthesis, its absence demonstrates that the mutant is truly LPS-deficient and not just making some incomplete precursor molecule with no antibody binding or LAL assay activity.

Although H44/76[pHBK30] is fully viable, a reduced growth rate compared to the wildtype strain was apparent. When grown overnight on GC agar plates, the mutant strain produced much smaller colonies; in liquid medium the doubling time during exponential growth was approximately 50% higher than in wildtype strain H44/76.

The morphology of H44/76[pHBK30] and its parent strain was examined by electron microscopy of ultrathin sections. In contrast to the wildtype, cells of H44/76[pHBK30] were more heterogeneous in size and a significant fraction showed signs of lysis. However, the outer membrane could be clearly discerned in the LPS-deficient mutant (fig.5). In contrast to the somewhat "baggy" appearance in the wildtype, the outer membrane of the mutant showed a "tighter fit", possibly indicating a lowered rate of synthesis.

Example 2: Construction of an *lpxA* knockout mutant

An *lpxA* knockout mutant of *N.meningitidis* was constructed by inserting a kanamycin-resistance cassette into the BstEII site located at position 293 within the *lpxA* gene of plasmid pLA21 (a pUC18 derivative with a 2.1 kb *lpxD-fabZ-lpxA* insert). The resulting plasmid pLAK33 was digested with *XbaI/SacI* and transformed to strain H44/76 with selection for kanamycin-resistance. As expected, the resulting colonies showed the same growth properties as the H44/76[pHBK30] mutant, indicating the lack of LPS. This was confirmed by a whole cell ELISA in which the *lpxA* knockout mutant did not bind any of the LPS-specific mAbs. These results

demonstrated once more that blocking of the lipid A biosynthesis pathway in *N. meningitidis* strain H44/76 leads to viable LPS-deficient mutants.

Detailed description of the methods and strains used in the examples.

5 Where no specific details are provided standard technology has been applied. Where references are provided the content thereof is to be considered incorporated herein.

Bacterial strains and plasmids

10 The *E.coli* strains NM522 and INVαF' were grown on LB medium at 37°C. The *N.meningitidis* strain H44/76 and its derivatives were grown at 37°C on GC medium base (Difco) supplemented with IsoVitaleX (Becton Dickinson) in a humid atmosphere containing 5% CO₂, or in liquid medium as described (van der Ley et al., 1993). For selection of meningococcal
15 transformants (van der Ley et al., 1996) kanamycin was used in a concentration of 75-100 µg/ml. With *E.coli*, antibiotics were used in the following concentrations: ampicillin, 100 µg/ml; kanamycin, 100 µg/ml. For cloning of PCR fragments, the TA cloning kit with the vector pCRII (Invitrogen) was used. Another vector used was pUC18.

20

Recombinant DNA techniques

Most recombinant DNA techniques were as described in Sambrook et al. (1989). Plasmid DNA was isolated using the pLAsmix kit (Talent). The polymerase chain reaction (PCR) was performed on a Perkin Elmer
25 GeneAmp PCR system 9600 with Taq polymerase. Sequence analysis was performed with an Applied Biosystems automatic sequencer on double-stranded plasmid DNA templates (isolated with Qiagen columns) and with a cycle sequencing protocol.

30 LPS analysis

Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed in 4% stacking and 16% separating gels as described by Lesse et al. (1990). Proteinase K-treated, boiled bacterial cells were used as samples. The gels were run for 17 h at a constant
35 current of 20 mA, and silver stained by the method of Tsai and Frasch (1982). The chromogenic LAL assay for endotoxin activity was performed using the QCL-1000 kit from BioWhittaker Inc. (Walkersville, MD, USA) according to the instructions of the manufacturer. Overnight cultures were diluted in meningococcal medium to an OD at 620 nm of 0.1, and

serial dilutions of these stocks were used as samples in the LAL assay. For fatty acid analysis by GC-MS, OMC samples were acetylated for 3 h at 90°C in pyridine and acetic acid anhydride in order to completely dissolve the LPS. The samples were subsequently heated for 3 h at 65°C in tetrahydrofuran in the presence of LiAlH₄ to reduce the O-linked fatty acids to the free alcohols. These were derivatized to TMS-ethers for 1 h at 60°C with BSTFA + 1% TMCS in pyridine, and analyzed by GC-MS on an Autospec (Micromass, Manchester, UK) in the electron impact mode. The amount of 3-OH C12 in the samples was quantified using 2-OH C12 as internal standard.

Characterization of OMP composition

Binding of mAbs specific for class 1, 3 and 4 OMPs and for the oligosaccharide part of immunotype L3 LPS was tested in a whole-cell ELISA (van der Ley et al., 1995, 1996). Isolation of OMCs by sarkosyl extraction and their analysis by SDS-PAGE were done as described previously (van der Ley et al., 1993).

LEGENDS TO THE FIGURES

Figure 1. Construction of H44/76[pHBK30]. Two-step PCR mutagenesis leading to the hybrid *lpxA* gene, with *E.coli*-specific primers Epr1 (ACT-GACGCGTGTTGATTGATAAATCCGC) seq. id. nr. 1 and Epr2 (GTAGGGCGGCACGTCCTGCGCCACACCGGA) seq. id. nr. 2 and *N.meningitidis*-specific primers Npr1 (TCCGGTGTGGCGCAGGACGTGCCGCCCTAC) seq. id. nr. 3 and Npr2 (CGGCCGCTCTAGAACTAGTGGATCA) seq. id. nr. 4.

Figure 2. Construction of H44/76[pHBK30]. Replacement of the chromosomal *lpxD-fabZ-lpxA* locus with the pHBK30 insert, carrying in addition to the *E.coli-N.meningitidis* hybrid *lpxA* gene a *kanR* selection marker instead of the 99 bp region between the MluI site in *fabZ* and the start codon of *lpxA*.

Figure 3. SDS-PAGE analysis of H44/76[pHBK30]. Silver-stained Tricine-SDS-PAGE LPS gel of proteinase K-treated whole-cell lysates of H44/76 (lanes 1 and 8) and six independent kanamycin-resistant transformants with pHBK30 (lanes 2-7).

Figure 4. SDS-PAGE of OMC proteins from H44/76[pHBK30] (lane 2) and H44/76 wildtype (lane 3); lane 1 contains a molecular weight marker of 94, 67, 43, 30, 20.1 and 14.4 kDa.

Figure 5. Electron micrograph of an H44/76[pHBK30] thin section, showing

the presence of the outer membrane in the absence of LPS.

REFERENCES

- 5 Akers, M.J. Parenteral Quality Control. Chapter 2: Pyrogen Testing. Marcel Dekker, Inc. New York and Basel, 1985, pp. 79-142.
- Ames, G. F.-L., Spudich, E.N. and Nikaido, H.: Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J. Bacteriol. 117 (1974) 406-416.
- 10 Anderson, M.S and Raetz, C.R.H: Biosynthesis of lipid A precursors in *Escherichia coli*. A cytoplasmic acyltransferase that converts UDP-N-acetylglucosamine to UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine. J. Biol. Chem. 262 (1987) 5159-5169.
- de Cock, H. and Tommassen, J.: Lipopolysaccharides and divalent cations are involved in the formation of an assembly-competent intermediate of outer-membrane protein PhoE of *E.coli*. EMBO J. 15 (1996) 5567-5573.
- Coleman, J. and Raetz, C.R.H.: First committed step of lipid A biosynthesis in *Escherichia coli*: Sequence of the *lpxA* gen e. J. Bacteriol. 170 (1988) 1268-1274.
- 20 Gabler, F.R. Pyrogens, and the depyrogenation of solutions with ultrafiltration membranes. In: Meltzer, T.H. Filtration in the pharmaceutical industry. Marcel Dekker, Inc. New York and Basel, 1987, pp. 919-939.
- 25 Karnovsky, M.J.: Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. Proc. 11th Ann. Meeting Soc. Cell Biol. 51 (1971) 146.
- Koplow, J. and Goldfine, H.: Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of *Escherichia coli*. J. Bacteriol. 117 (1974) 527-543.
- 30 Kulshin, V.A., Zähringer, U., Lindner, B., Frasch C.E., Tsai, C., Dimitriev, A. and Rietschel, E.T.: Structural characterization of the lipid A component of pathogenic *Neisseria meningitidis*. J. Bacteriol. 174 (1992) 1793-1800.
- 35 Laird, M.W., Kloser, A.W. and Misra, R.: Assembly of LamB and OmpF in deep rough lipopolysaccharide mutants of *Escherichia coli* K-12. J. Bacteriol. 176 (1994) 2259-2264.
- Lesse, A.J., Campagnari, A.A., Bittner, W.E. and Apicella,

- M.A.: Increased resolution of lipopolysaccharides and lipooligosaccharides utilising tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Immunol. Meth.* 126 (1990) 109-117.
- 5 van der Ley, P., van der Biezen, J., Hohenstein, P., Peeters, C. and Poolman, J.T.: Use of transformation to construct antigenic hybrids of the class 1 outer membrane protein in *Neisseria meningitidis*. *Infect. Immun.* 61 (1993) 4217-4224.
- 10 van der Ley, P., van der Biezen, J. and Poolman, J.T.: Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the *porA* gene for use in the production of a multivalent outer membrane vesicle vaccine. *Vaccine* 13 (1995) 401-407.
- 15 van der Ley, P., Kramer, M., Steeghs, L., Kuipers, B., Andersen, S.R., Jennings, M.P., Moxon, E.R. and Poolman, J.T.: Identification of a locus involved in meningococcal lipopolysaccharide biosynthesis by deletion mutagenesis. *Mol. Microbiol.* 19 (1996) 1117-1125.
- 20 Nakano, M. and Matsuura, M.: Lipid A. In: Stewart-Tull, D.E.S. (Ed.), *The Theory and Practical Application of Adjuvants*. John Wiley & Sons, 1995, pp. 315-335.
- Onishi, H.R., Pelak, B.A., Gerckens, L.S. et al.: Antibacterial agents that inhibit lipid A biosynthesis. *Science* 274 (1996) 980-982.
- 25 Poolman, J.T.: Development of a meningococcal vaccine. *Infect. Agents Dis.* 4 (1995) 13-28.
- Raetz, C.R.H.: Biochemistry of endotoxins. *Annu. Rev. Biochem.* 59 (1990) 129-170.
- 30 Raetz, C.R.H. and Roderick, S.L.: A left-handed parallel β helix in the structure of UDP-N-Acetylglucosamine acyltransferase. *Science* 270 (1995) 997-1000.
- Reid, G., Hindennach, I. and Henning, U.: Role of lipopolysaccharide in assembly of *Escherichia coli* outer membrane proteins OmpA, OmpC and OmpF. *J. Bacteriol.* 172 (1990) 6048-6053.
- 35 Sambrook, J., Fritsch, E.F. and Maniatis, T.: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
- Sen, K. and Nikaido, H.: Lipopolysaccharide structure required for in vitro trimerization of *Escherichia coli* OmpF porin. *J. Bacteriol.* 173 (1991) 926-928).

- Steeeghs, L., Jennings, M.P., Poolman, J.T. and van der Ley, P.: Isolation and characterization of the *Neisseria meningitidis* *lpxD-fabZ-lpxA* gene cluster involved in lipid A biosynthesis. *Gene* 190 (1997) 263-270.
- 5 Tsai, C.M. and Frasch, C.E.: A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119 (1982) 115-119.
- Verheul, A.F.M., Snippe, H. and Poolman, J.T.: Meningococcal lipopolysaccharides: Virulence factor and potential vaccine component. *Microbiol. Rev.* 57 (1993) 34-49.
- 10 Vuorio, R., Härkönen, T., Tolvanen, M. and Vaara, M.: The novel hexapeptide motif found in the acyltransferases LpxA and LpxD of lipid A biosynthesis is conserved in various bacteria. *FEBS Lett.* 337 (1994) 289-292.
- 15 Zähringer, U., Lindner, B. and Rietschel, E.Th.: Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. In: Horton, D. (Ed.), *Advances in Carbohydrate Chemistry and Biochemistry*, vol.50. Academic Press Inc, San Diego, 1994, pp. 211-276.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

(A) NAME: De Staat der Nederlanden

(B) STREET: P.O. box 1

(C) CITY: Bilthoven

10

(D) STATE: Utrecht

(E) COUNTRY: The Netherlands

(F) POSTAL CODE (ZIP): 3720 BA

15 (ii) TITLE OF INVENTION: Novel mutants of gramnegative mucosal
bacteria and application thereof in vaccines.

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

20

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

25

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(vi) ORIGINAL SOURCE:

(A) ORGANISM: E. coli

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

15

ACTGACGCGT GTGATTGATA AATCCGC

27

(3) INFORMATION FOR SEQ ID NO: 2:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: E. coli

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTAGGGCGGC ACGTCCTGCG CCACACCGGA

30

20

(4) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
25 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: N. meningitidis

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCCGGTGTGG CGCAGGACGT GCCGCCCTAC

30

(5) INFORMATION FOR SEQ ID NO: 4:

16

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

5 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: *N. meningitidis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

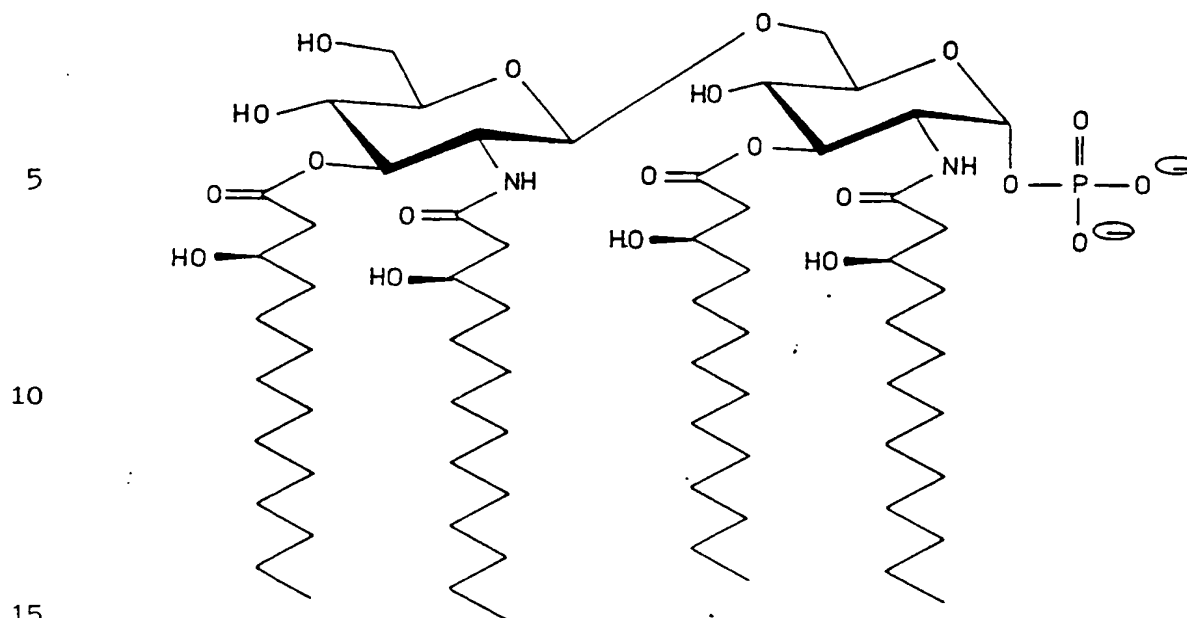
15 CGGCCGCTCT AGAACTAGTG GATCA

25

CLAIMS

1. Gram negative mucosal bacterium comprising a mutation such that it is viable, capable of OMP formation and lacks endotoxic lipopolysaccharide (LPS).
5
2. Gram negative mucosal bacterium according to claim 1 substantially free of LPS.
- 10 3. Gram negative mucosal bacterium according to claim 1 or 2 comprising a mutation such that it is substantially free of Lipid A.
4. Gram negative mutant according to any of the preceding claims, said bacterium being selected from the group comprising diplococci.
15
5. Gram negative mutant according to any of the preceding claims, said bacterium being selected from the group comprising gonococci, e.g. *N.gonorrhoeae*.
- 20 6. Gram negative mutant according to any of claims 1-4, said bacterium being selected from the group comprising meningococci e.g. *N.meningitidis*.
7. Gram negative mutant according to any of claims 1-3, said
25 bacterium being selected from the group comprising *Bordetella*, e.g. *Bordetella pertussis*.
8. Gram negative mutant according to any of the preceding claims, said mutant comprising a mutation in at least one gene associated with
30 Lipid A biosynthesis.
9. Gram negative mutant according to any of the preceding claims, said mutant comprising a mutation in at least one gene associated with the early stage of Lipid A biosynthesis, said early stage being prior to
35 formation of the following structure

18



10. Gram negative mutant according to any of the preceding claims, said mutant comprising a mutation in at least one gene selected from the group comprising *lpxA*, *lpxD* and *lpxB*.

20 11. Gram negative mutant according to any of the preceding claims, said mutant comprising a mutation in at least the gene *lpxA*.

12. Attenuated live vaccine against a gram negative mucosal bacterium said vaccine comprising a mutant according to any of the
25 abovementioned claims as active component and a pharmaceutically acceptable carrier.

13. Whole cell vaccine against a gram negative mucosal bacterium said vaccine comprising a mutant according to any of the claims 1-11 as
30 active component and a pharmaceutically acceptable carrier.

14. OMP Vaccine against a gram negative mucosal bacterium said vaccine comprising OMP derived from a mutant according to any of claims
35 1-11 as active component and a pharmaceutically acceptable carrier.

15. Vaccine according to any of claims 12-14 further comprising an adjuvant.

16. Vaccine according to any of the claims 12-15 said vaccine being

16. Vaccine according to any of the claims 12-15 said vaccine being substantially free of endotoxic LPS, wherein substantially free is defined as LPS free according to the Limulus assay.

5 17. Vaccine according to claim 16 free of LPS.

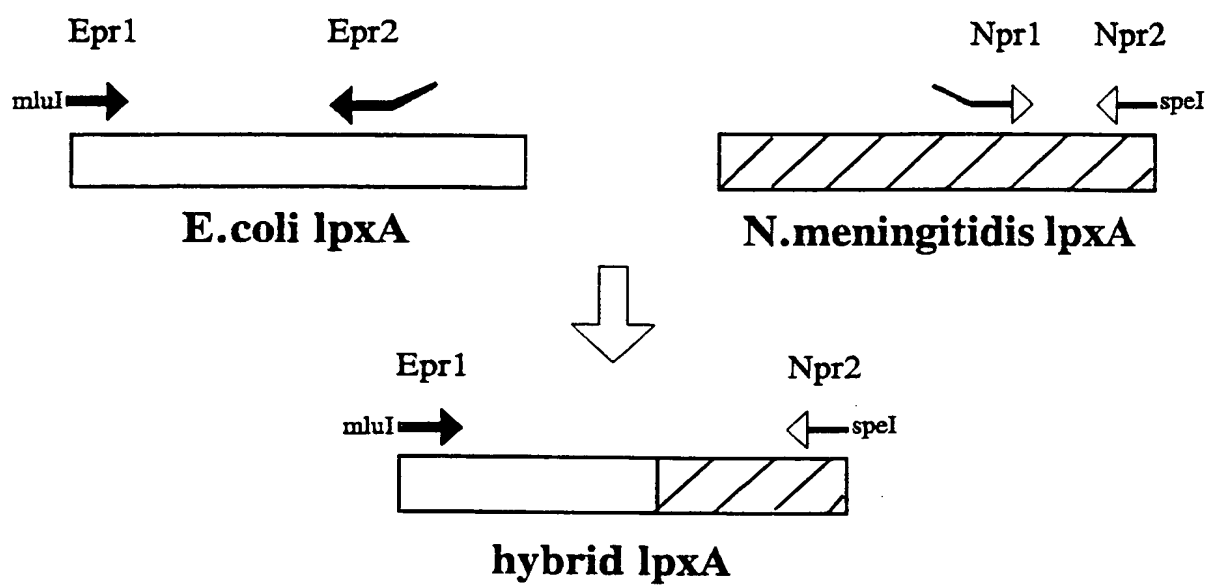
18. A method of producing LPS free vaccine comprising application of a mutant according to any of claims 1-11 and/or a part derived from said mutant as active component of a vaccine in a manner known per se for
10 preparing vaccine formulations, said method being free of measures to remove LPS by purification.

19. A method of producing LPS free OMP comprising culturing a mutant according to any of claims 1-11 and deriving an OMP comprising
15 fraction from said culture in a manner known per se for isolating protein from bacterial culture, said method being free of additional measures to remove LPS from said culture or OMP comprising fraction.

20. OMP substantially free of LPS.

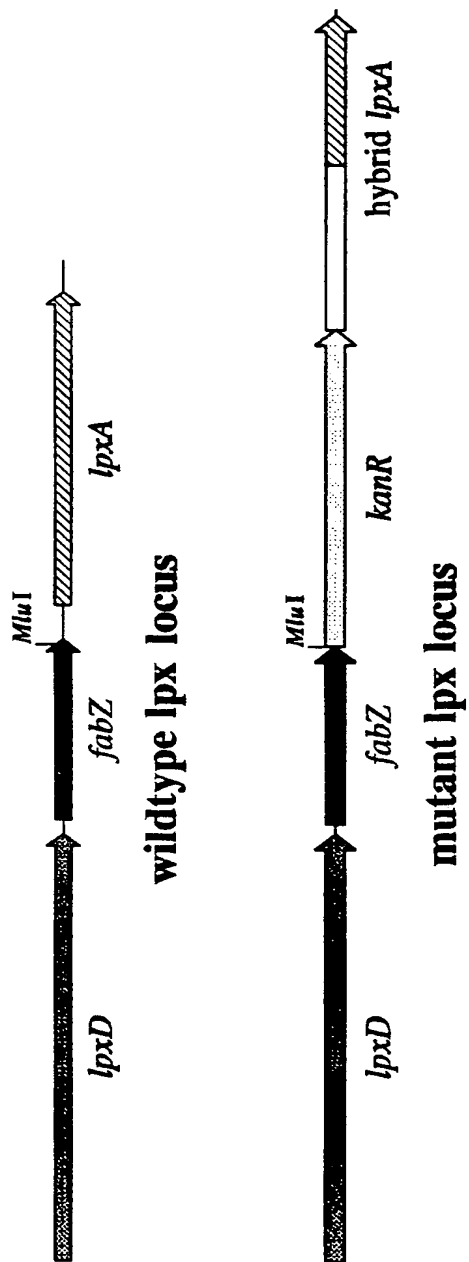
1/5

fig -1

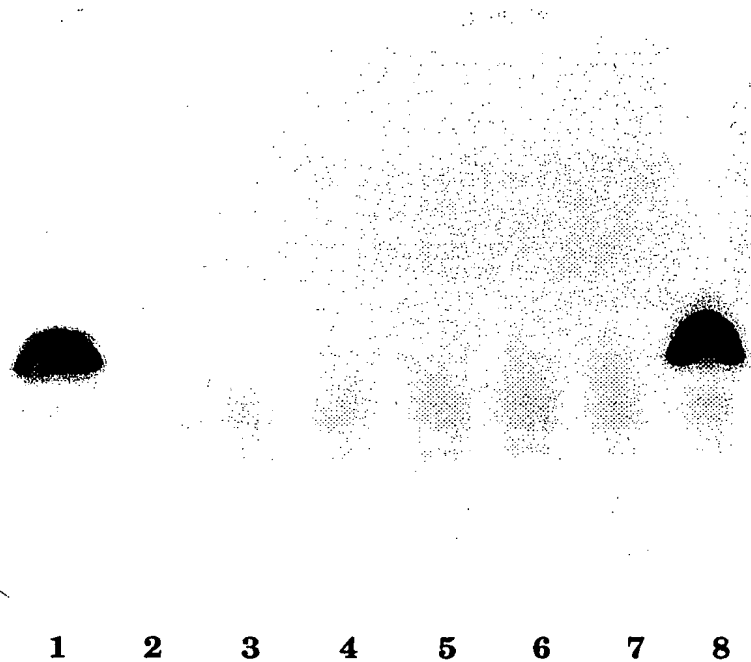


2/5

fig-2

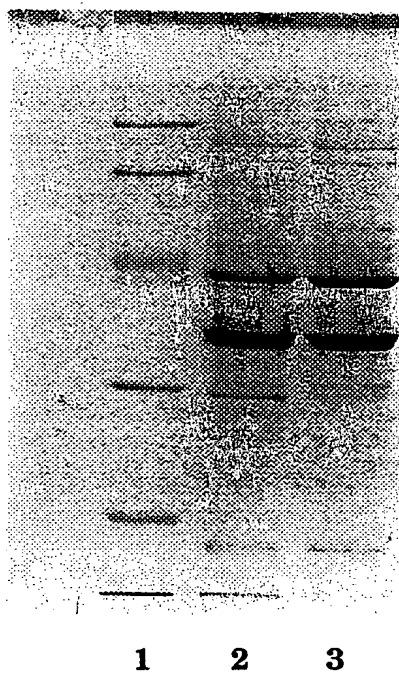


Figuur 3



4/5

Figuur 4



Figuur 5



INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 97/00474

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N1/20 A61K39/02 A61K39/095 C07K14/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25061 A (SQUIBB BRISTOL MYERS CO ; SOMERVILLE JOHN E JR (US); DARVEAU RICHAU) 17 July 1997 see page 3, line 32 - page 5, line 20 ---	1,7,8, 12,13, 15,16
X	WO 97 19688 A (UNIV IOWA RES FOUND ;UNIV CALIFORNIA (US); AMERICAN CYANAMID CO (U) 5 June 1997 see page 4, line 10 - page 5, line 5 see page 6, line 29 - page 7, line 7 ---	1,4-6,8, 12,13, 15,16
X	EP 0 624 376 A (AMERICAN CYANAMID CO) 17 November 1994 see page 2, line 45 - page 3, line 21 --- -/--	14-17,20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

6 May 1998

Date of mailing of the international search report

26/05/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 97/00474

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>STEEGHS ET AL: "ISOLATION AND CHARACTERIZATION OF THE NEISSERIA MENINGITIDIS LPXD-FABZ-LPXA GENE CLUSTER INVOLVED IN LIPID A BIOSYNTHESIS" GENE, vol. 190, no. 2, 6 May 1997, pages 263-270, XP002064116 cited in the application see the whole document</p> <p style="text-align: center;">---</p>	
A	<p>ODEGAARD ET AL: "SHORTENED HYDROXYACYL CHAINS ON LIPID A OF ESCHERICHIA COLI CELLS EXPRESSING A FOREIGN UDP-N-ACETYLGLUCOSAMINE O-ACYLTRANSFERASE" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 32, 8 August 1997, pages 19688-19696, XP002064117 see page 19688 see abstract</p> <p style="text-align: center;">---</p>	
A	<p>RAETZ ET AL: "BIOCHEMISTRY OF ENDOTOXINS" ANNUAL REVIEWS OF BIOCHEMISTRY, vol. 59, 1990, pages 129-170, XP002064118 cited in the application see page 147, paragraph 4 - page 148, paragraph 3</p> <p style="text-align: center;">-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter: International Application No

PCT/NL 97/00474

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725061 A	17-07-97	NONE	
WO 9719688 A	05-06-97	AU 1124697 A	19-06-97
EP 0624376 A	17-11-94	CA 2123355 A	14-11-94
		JP 8019396 A	23-01-96